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RDX Biodegradation by a Methanogenic Enrichment Culture Obtained from an Explosives Manufacturing Wastewater Treatment Plant

by
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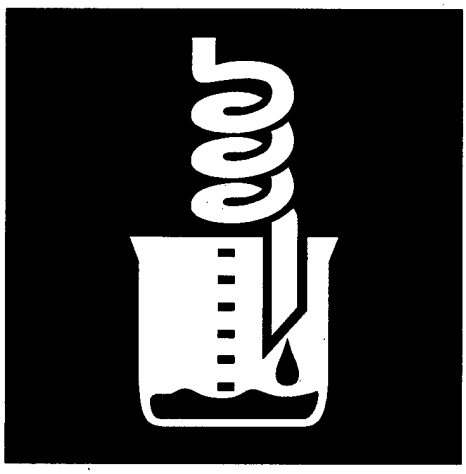

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13. ABSTRACT (Maximum 200 words)

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Foreword

This study was conducted for the Directorate of Military Programs, Headquarters, U.S. Army Corps of Engineers (HQUSACE), under Project 4A161102AH68, "Processes in Pollution Abatement Technology"; Work Unit FKL, "Pathways and Controlling Factors in Biodegradation of Energetic Wastes."

The work was performed by the Industrial Operations Division (UL-I) of the Utilities and Industrial Operations Laboratory (UL), U.S. Army Construction Engineering Research Laboratories (CERL). The CERL principal investigator was Neal R. Adrian. Walter J. Mikucki is Chief, CECER-UL-I; John T. Bandy is Operations Chief, CECER-UL; and Gary W. Schanche is the responsible Technical Director, CECER-TD. The CERL technical editor was William J. Wolfe, Technical Resources.

Dr. Michael J. O'Connor is Director of CERL.

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1 Introduction

Background

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Figure 1) is the most important military high explosive in the United States today (Gorontzy et al. 1994). RDX is manufactured at the Holston Army Ammunition Plant (AAP) in Kingsport, TN. Wastewater produced from the manufacture of RDX is treated on site in an industrial wastewater treatment plant and discharged to the Holston River. RDX is also a frequent component of pink water, a hazardous wastewater generated from: (1) load, assembly, and packaging (LAP) of conventional ammunition items, and (2) demilitarization operations where explosives are washed out of disassembled ammunition (Concurrent Technologies Corporation 1996). Pinkwater is typically treated using granular activated carbon, although potentially less costly alternatives are under investigation (Concurrent Technologies Corporation 1996). One such alternative for pink water and other wastewater contaminated with nitroaromatic compounds is biological treatment using an anaerobic fluidized-bed granular activated carbon bioreactor (Concurrent Technologies Corporation 1996).

In the past, improper disposal of wastewater has led to environmental contamination. Recent reports estimate that at least 28 U.S. Army installations (Funk et al. 1993) and 200 areas in Germany contain soils contaminated with high explosives, including RDX (Binks, Nicklin, and Bruce 1995). Many of these sites have the potential to contaminate groundwater.

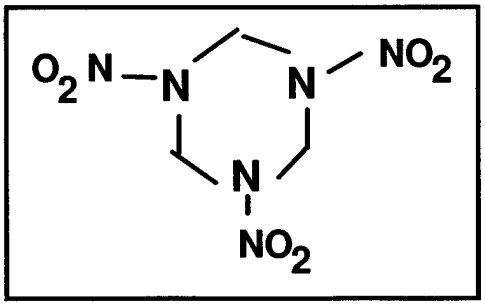

Figure 1. Molecular structure of the nitramine explosive RDX.

Despite the Army's need for information on the anaerobic biodegradation of explosives, relatively little is known (Gorontzy et al. 1994). RDX is reported to be more easily biodegraded under anaerobic, rather than aerobic conditions (Funk et al. 1993; Kitts, Cunningham, and Unkefer 1994; McCormick, Cornell, and Kaplan 1981; Roberts, Ahmad, and Pendharkar 1996). The few exceptions include RDX biodegradation by a white rot fungus (Fernando and Aust 1991), by the bacterium *Stenotrophomonas maltophilia* PB1 when using RDX as the sole source of nitrogen (Binks, Nicklin, and Bruce 1995), and during composting of explosives contaminated soil (Williams, Ziegenfuss, and Sisk 1992).

Most of the studies demonstrating RDX biodegradation under anaerobic conditions were conducted in poorly defined environments where the electron donor and acceptors were not firmly established. For example, in three such studies, the bacterial cultures were grown in nutrient broth (McCormick, Cornell, and Kaplan 1981), yeast extract (Kitts, Cunningham, and Unkefer 1994), and Brain Hear Infusion media (Regan and Crawford 1994). In the latter two cases, RDX biodegradation was carried out by pure cultures of bacteria isolated from explosives-contaminated soil. In the former case, the nutrient broth was inoculated with organisms from activated sludge.

Several studies have been carried out under nitrate-reducing conditions (Bell, Burrows, and Carrazza 1987; McCormick, Cornell, and Kaplan 1984). The results, however, are inconclusive. While RDX degradation and nitrate removal occurred in the experimental systems studied, it is not clear whether RDX degradation and nitrate depletion occurred simultaneously. Further research is required to determine if RDX degradation is linked to nitrate-reduction.

Objectives

The primary objectives of this part of the study were to investigate RDX biodegradation under rigorously controlled anaerobic conditions and to obtain a RDX biodegrading enrichment culture. This study focused on the biodegradation of RDX by a methanogenic enrichment culture derived from an explosives manufacturing wastewater treatment plant. This is the first report demonstrating RDX biodegradation under methanogenic conditions.

Approach

1. A literature review was conducted of laboratory and field studies involving the biodegradation of RDX. The analyzed information indicated that RDX was susceptible to biodegradation under anaerobic conditions, but recalcitrant under aerobic conditions. Under anaerobic conditions, however, little is known about the specific conditions, making it difficult to extrapolate these findings to the field. The literature review identified areas needing further research. These areas are detailed in this report.

2. RDX was obtained from Holston AAP.

3. Biodegradation studies were carried out in serum bottles using sludge and wastewater obtained from the treatment plant at Holston AAP.

4. Enrichment cultures were obtained by re-amending serum bottles with RDX after its degradation, and by adding ethanol or butyrate as an electron donor.

5. Liquid samples were taken periodically from the serum bottles and analyzed.

6. RDX and biodegradation intermediates were analyzed by high pressure liquid chromatography.

7. The results were analyzed and conclusions were drawn based on the results of this stage of work.

Mode of Technology Transfer

Findings from this research will be incorporated into ongoing Exploratory Development (6.2) work in treatment of munitions wastewater.

2 Materials and Methods

Chemicals

RDX used in experiments was obtained from the Holston Army Ammunition Plant (93.8 to 99.6 percent pure). RDX analytical standards were obtained from a commercial vendor (AccuStandard, Inc., New Haven, CT). All other chemicals were obtained from major suppliers of chemicals and were of the highest purity obtainable.

Source of Inoculum

Biodegradation studies were carried out in serum bottles using sludge and wastewater obtained from an industrial wastewater treatment plant located at the Holston Army Ammunition Plant. The treatment plant receives wastewater contaminated with RDX and HMX. Samples were collected from the beginning segment of the treatment plant known as the anoxic filter and stored at 4 °C until use.

Serum Bottle Biodegradation Studies

Biodegradation of RDX was evaluated by comparing substrate disappearance in the experimental bottles to that in sterile controls. Microcosms were prepared by making a stock solution of RDX in acetonitrile and adding it to sterile 160 ml serum bottles. The acetonitrile was allowed to evaporate overnight, leaving behind a thin layer of RDX. Eighty ml of a sterile (steam sterilization, 121 °C, 15 min) basal salts medium containing resazurin (0.0002 percent) was added to the serum bottles, followed by the addition of 20 ml of a wastewater-sludge slurry containing 5 grams wet sludge. The basal salts solution consisted of the following quantity per liter: NaCl, 0.8 g; NH_4Cl, 1.0 g; KCl, 0.1 g; $MgSO_4 \bullet 7H_2O$, 0.02 g; KH_2PO_4, 1.35; K_2HPO_4, 1.75 g; $NaHCO_3$, 1.0 g; trace metal solution, 10 ml; vitamins, 10 ml. Trace metal and vitamin solutions were made as previously described (Tanner, McInerney, and Nagle 1989). The pH of the medium was

adjusted to 7.2. The basal salts medium refers to the basal salts containing trace metals and vitamins. The medium was prepared and dispensed using strict anoxic techniques as previously described (Shelton and Tiedje 1984). After addition of the slurry, the bottles were sealed with black butyl stoppers and aluminum crimp seals. The headspace of the bottles was evacuated and replaced with a mixture of N_2:CO_2 (80:20) three times, and then pressurized to 1.3 ATM. Sterile controls were prepared by taking serum bottles prepared as described above, with the exception that they were not amended with RDX. They were autoclaved on 3 successive days (121 °C, 15 min). Then the contents were transferred to a sterile serum bottle containing RDX. The bottles were then sealed as previously described. The study was conducted in triplicate.

Enrichment Culture Studies

A methanogenic enrichment culture was obtained by re-amending serum bottles with RDX after its degradation and adding ethanol or butyrate as an electron donor. After several additions of RDX, the enrichment was periodically transferred (20 to 40 percent) to fresh basal salts medium. Studies with the enrichment culture were done in smaller serum bottles (35 ml volume). A 200 μM RDX stock solution in deionized water was made and shaken overnight at 35 °C. Approximately 17 ml of the enrichment culture was added to sterile, nitrogen flushed serum bottles. Three ml of the RDX stock solution were added to the serum bottles to reach a target concentration of approximately 30 μM RDX. Studies were conducted in triplicate at room temperature. Strict anaerobic techniques were used during media preparation, culturing, and sampling.

Analytical Methods

RDX and biodegradation intermediates were analyzed by high pressure liquid chromatography (HPLC) using a Waters Module 1 HPLC System outfitted with a Lichrosphere C-18 reverse phase column (250 mm x 4.6 mm, 5 μm; Alltech Associates, Inc., Deerfield, IL). The following HPLC conditions were used: mobile phase, 55:45 (methanol:50 mM acetate buffer, pH 4.5); injection volume, 20 ul; flow rate, 1.1 ml/minute; wavelength, 254 nm. Later analyses employed an acetonitrile:acetate buffer (45:55%, v/v) mobile phase. Identification of unknown biodegradation intermediates was carried out by comparing their retention time with those of authentic standards.

The headspace of the serum bottles was monitored for the formation of CH_4 by gas chromatography. Methane produced from unamended controls was subtracted from that produced in substrate-amended bottles. This amount was compared to the theoretically expected amount of CH_4 (Gottschalk 1986; McInerney 1986). Gas samples were injected into a SRI gas chromatograph equipped with a flame ionization detector and a Porapak-Q packed column (Alltech Associates, Inc., Deerfield, IL). The GC conditions were: helium flow rate, 30 ml/min; injector, oven, and detector temperatures, 75 °C.

Sampling

Liquid samples were taken periodically from the serum bottles using a syringe and needle and were stored at –20 °C until use. Samples were centrifuged at 12,000 x g for 4 minutes in a bench top microcentrifuge before analyzing by HPLC. Methane concentrations were determined by taking samples of the headspace gas (0.2 ml) using a 1 ml disposable syringe with a 21 ga needle, and then injecting the samples directly into the GC as described above.

3 Results and Discussion

Serum Bottle Biodegradation Studies

RDX was biodegraded in serum bottles incubated under methanogenic conditions (Figure 2). In these bottles, three transient appearing peaks were observed in the chromatograms during HPLC analysis of the aqueous samples (Figure 2). These compounds were identified as mononitroso-RDX, dinitroso-RDX, and trinitroso-RDX by comparing their HPLC retention times with authentic standards (data not shown). After approximately 27 days, the compounds were no longer detected in the serum bottles. The purity of the standards used to identify the intermediates were unknown, therefore researchers were unable to quantitate them. However, Figure 2 shows the relative area of the peak on the chromatogram corresponding to the compound of interest. Although no other intermediates were observed in the HPLC chromatograms, the biodegradation of RDX was hypothesized to proceed in a manner similar to that proposed by McCormick, Cornell, and Kaplan (1981), who reported that the RDX molecule is destabilized and spontaneously fragments when the nitro groups are reduced to the hydroxylamino level. Others (Kitts, Cunningham, and Unkefer 1994) have also reported ring cleavage of the RDX molecule.

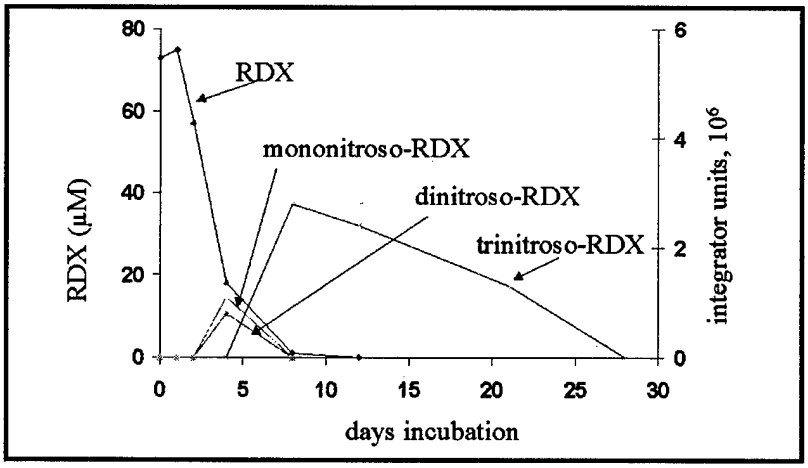

Figure 2. RDX biodegradation in serum bottles incubated under methanogenic conditions. Three transient appearing peaks were identified as the mono-, di-, and trinitroso-RDX intermediates. The integrator units of the peak corresponding to the nitroso-intermediate is shown.

Table 1. Methane production in serum bottles containing wastewater from an explosives manufacturing wastewater treatment plant. The bottles were amended with RDX to approximately 80 μM.

Sample	Methane (μmoles) Initial	Final	Methane (μmoles) Formed
Sterile Control	0	0	0
Unamended Control	0	40	40
RDX Amended	0	3	3

The presence of RDX inhibited methane production (Table 1). For example, only 3 μmoles methane was produced in bottles amended with RDX, compared to 40 μmoles in RDX unamended controls. Inhibition of methane production by RDX was not surprising since RDX is known to be toxic to aquatic microorganisms (Drzyzga et al. 1995). Furthermore, nitroaromatic compounds can lyse methanogenic bacteria and inhibit methane formation in anaerobic sewage sludge (Gorontzy, Kuver, and Blotevogel 1993). TNT is also known to be mutagenic and toxic to microorganisms (Roberts, Ahmad, and Pendharkar 1996). Although previous reports suggest that the toxicity of RDX may be responsible for the inhibition on methane production, subsequent studies (described below) suggest that the inhibition is due to something other than the toxicity.

Enrichment Culture Studies

The initial RDX degradation rate in bottles incubated under methanogenic conditions in the screening studies was 9 μM day^{-1}. Attempts were made to enrich for this activity by re-amending the bottles with RDX when it was no longer detected in the aqueous phase. Despite these efforts, the RDX biodegradation activity decreased over time. Ethanol and butyrate were added to the bottles in an attempt to sustain the activity. The RDX degradation rates in bottles amended with butyrate and water were about 2 μM day^{-1} and decreasing, while the degradation rate in the ethanol amended culture had stabilized at 4 μM day^{-1} (data not shown). The contents of the serum bottle was periodically transferred to fresh basal salts medium (10 to 20 percent by volume) and were amended with ethanol and RDX when they were depleted. After several transfers, the contents of the serum bottle was transferred to a 1-L bottle. Ethanol, RDX, and basal salts medium were periodically added. This enrichment culture was used in all subsequent studies.

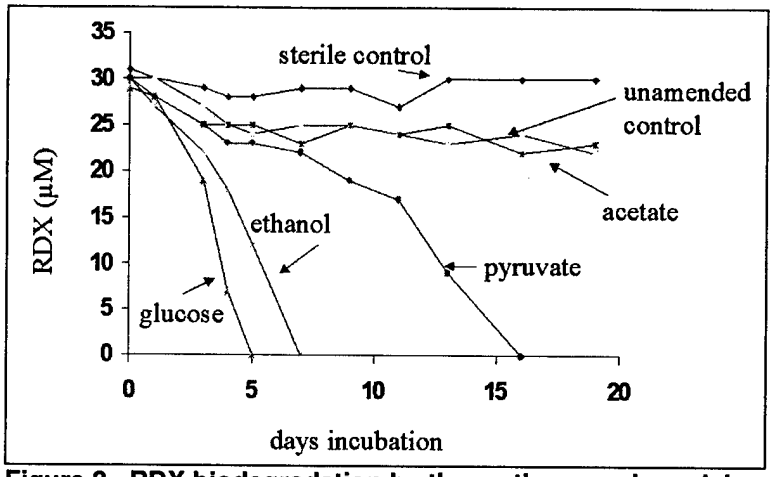

Figure 3. RDX biodegradation by the methanogenic enrichment culture when amended with acetate, ethanol, pryuvate, or glucose. The electron donors were added to a concentration of 1 mM.

RDX was biodegraded by the enrichment culture when ethanol, pyruvate, or glucose were supplied as electron donors (Figure 3). Glucose and ethanol appeared to be better cosubstrates than pyruvate for supporting RDX biodegradation. After 7 days, RDX was no longer detected in serum bottles amended with glucose or ethanol, while in bottles amended with pyruvate >70 percent of the RDX remained. In the latter case, RDX was eventually depleted after 16 days. Interestingly, acetate did not support RDX biodegradation (Figure 3). There was no loss of RDX in cosubstrate unamended and sterile controls throughout the study period.

Several electron donors supported methane production when added to the enrichment culture, but the addition of RDX stopped methane production. For example, 5.2 μmoles methane were produced when ethanol was added to the enrichment culture, but only 0.2 μmoles were produced when RDX was added along with the ethanol (Table 2), for a 96 percent decrease in the amount of methane produced. RDX had a similar effect on methane production when glucose was the cosubstrate. Bottles amended with RDX showed a 91 percent decrease in methane production compared to the RDX unamended controls (Table 2). Acetate and pyruvate did not support methane production (Table 2). Methane production in these bottles was similar to the cosubstrate unamended control. Less than 25 percent of the methane predicted from the complete mineralization of ethanol to CH_4 and CO_2 was observed in RDX unamended controls (Table 3). However, acetate, an intermediate produced during the mineralization of ethanol to CH_4 and CO_2, did not support methane production by the enrichment culture (Table 2).

Table 2. Inhibition of methane production in the enrichment culture by RDX. The bottles were amended to a concentration of 1 mM with the respective electron donor and 25 μM RDX. Reported methane values (in μmoles) were taken after 19 days incubation.

Electron Donor	Methane Produced	
	RDX Amended	RDX Unamended
none	[a]ND	0.4
ethanol	0.2	5.2
glucose	0.2	2.2
acetate	0.2	0.6
pyruvate	0.1	0.3

[a] not done

Table 3. Methane recovery from 1 mM ethanol when added to the enrichment culture.

Ethanol (mM)	μmoles Ethanol	Expected Methane (μmoles)	Observed Methane (μmoles)	% Recovered
0	0.0	0.0	0.7	[a]NA
1	17.3	[b]25.9	6.0	23
1	17.3	[c]8.6	6.0	70

[a] Not applicable
[b] Calculated according to the following equation: 2 ethanol ----> CO_2 + 3 CH_4
[c] Calculated according to the following equation: 2 ethanol + HCO_3^- ---> 2 acetate + CH_4 + H_2O + H^+

Furthermore, subsequent studies demonstrated that acetate accumulates in the medium and is apparently not used by the enrichment culture (data not shown). When acetate's contribution to methane production is subtracted, 70 percent of the expected amount of methane from ethanol was observed (Table 3).

However, the loss of methane production when the enrichment culture was fed RDX along with ethanol (Table 2) is still unexplained. Since the enrichment culture could not use acetate, the proposed reactions for ethanol utilization by the enrichment culture are:

	Reaction	Equation		$\Delta G^{\circ\prime}$ (kJ)	
	ethanol + H_2O	↔	Acetate$^-$ + H^+ + 2 H_2	+ 9.6	Eq 1
	4 H_2 + HCO_3^- + H^+	↔	CH_4 + 3 H_2O	- 135.6	Eq 2
Net:	2 ethanol + HCO_3^-	↔	2 acetate- + CH_4 + H_2O + H^+	-116.4	Eq 3

This pathway is consistent with known reactions occurring under methanogenic conditions with a consortium of bacteria (McInerney 1986). RDX is hypothesized to serve as a hydrogen sink, diverting hydrogen away from methane production

(Eq 2). In other words, H_2 produced during the metabolism of ethanol was used to reduce RDX, not CO_2, thus suppressing methane production. To test this hypothesis, increasing concentrations of ethanol (10 mM) were added to the enrichment culture to ensure that more H_2 was produced than needed for RDX biodegradation. As predicted, no methane was produced in the presence of RDX, but after its depletion, methane production started (Figure 4). It appears that, when RDX is no longer present, the H_2 produced by the bacteria (Eq 1) becomes available to the methanogens and methane production resumes (Eq 2). There was no lag in the methane production in RDX unamended controls. These observations support the hypothesis that RDX serves as an H_2 sink, diverting H_2 away from methane production. Boopathy and Kulpa have suggested a similar phenomenon with sulfate-reducing bacteria and nitroaromatic compounds (Boopathy and Kulpa 1993). They reported nitroaromatic compounds may substitute for sulfate as catabolic electron acceptors for *Desulfovibrio sp.* (B strain).

Other evidence supports our contention that RDX is serving as a terminal electron acceptor in methanogenic environments. For example, the metabolism of ethanol to acetate and H_2 is not thermodynamically favorable (+9.6 kJ), as shown in Eq 1. Ethanol metabolism only proceeds if one of the products is removed, resulting in a thermodynamically favorable reaction. Typically the methanogens remove H_2 (Eq 2), pulling Eq 1 forward. The sum of the two reactions results in an overall thermodynamically favorable reaction for the metabolism of ethanol to acetate and CH_4 (Eq 3). Methane production is inhibited when bromoethanesulfonic acid (BESA), an inhibitor of methanogenic bacteria, is added to the serum bottles (Figure 5). The partial pressure of H_2 increases and ethanol degradation stops, as predicted by Eq 1. RDX biodegradation should therefore also cease. Interestingly, this did not occur. Figure 6 demonstrates that BESA had no effect on RDX biodegradation. The metabolism of ethanol continued, supplying H_2 for the reduction of RDX. Furthermore, greater than 50 percent of the predicted amount of methane from ethanol was observed, compared to less than 5 percent in the presence of BESA or RDX (Table 4). RDX appears to be replacing CO_2 as an H_2 sink during ethanol degradation since the metabolism of ethanol to acetate without H_2-using methanogens is not thermodynamically feasible (McInerney 1986).

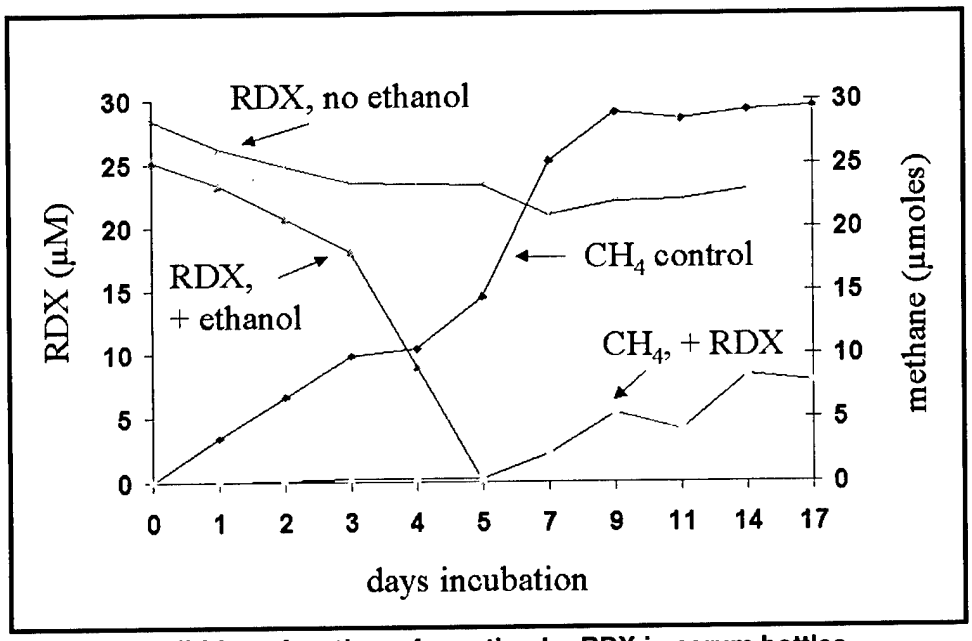

Figure 4. Inhibition of methane formation by RDX in serum bottles amended with 10 mM ethanol. The RDX concentrations are also shown.

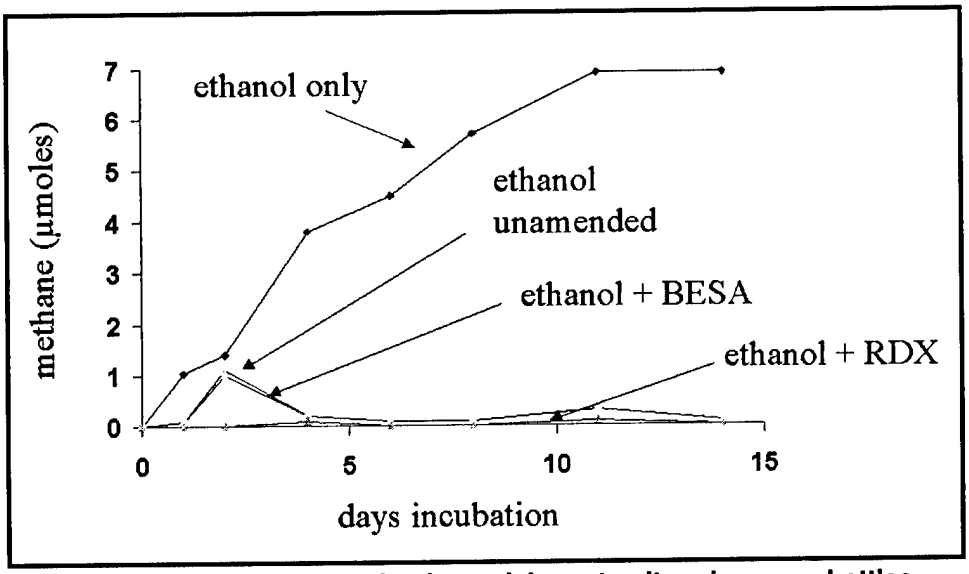

Figure 5. Methane formation by the enrichment culture in serum bottles amended with RDX and BESA containing 2 mM ethanol.

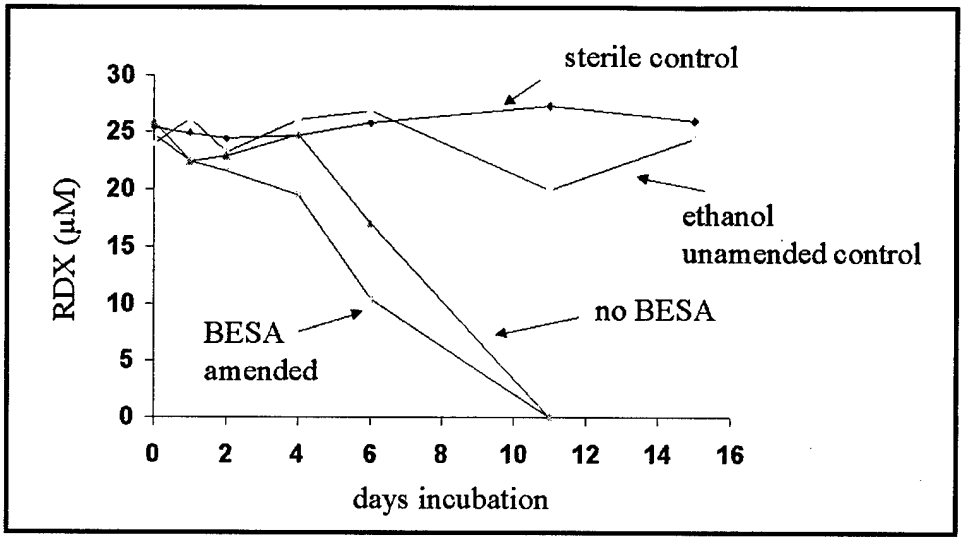

Figure 6. Biodegradation of RDX by the methanogenic enrichment culture in serum bottles amended with BESA and 2 mM ethanol. The RDX concentrations in the sterile and ethanol unamended controls are also shown.

Table 4. Methane formation from ethanol by the enrichment culture in bottles amended with RDX or BESA. Ethanol and RDX were added to a concentration of 2 mM and 25 μM, respectively.

Sample	μmoles Ethanol	[a]Stoichiometry (Mole CH_4 per Mole Substrate)	CH_4 (μmoles) Expected	CH_4 (μmoles) Formed	% CH_4 Recovered
No ethanol	0	—	0	<1	NA
No BESA	30	0.5	15	8	>50
+ BESA	40	0.5	20	<1	<5
+ RDX	40	0.5	20	<1	<5

[a]calculated according to the following equation: 2 ethanol + HCO_3^- -- >2 acetate + CH_4 + H_2O + H^+

4 Conclusions

This study has shown that RDX obtained from the Holston Army Ammunition wastewater treatment plant was biodegraded by a methanogenic enrichment culture when fed reduced cosubstrates such as ethanol. RDX served as an terminal electron sink, diverting H_2 away from carbon dioxide reduction, thus inhibiting methane production.

Initial studies showed mononitroso-, dinitroso-, and trinitroso-RDX intermediates. This suggests that the biodegradation pathway is similar to that reported by Kaplan et al. No transient intermediates were seen in later studies with the enrichment culture, but the same pathway is likely used. The absence of intermedates is likely due to the much lower concentration of RDX (30 μM) compared to concentrations of 80 μM or more in the initial studies of this project. More work is needed to clarify the biodegradation pathway, especially regarding products formed after reduction of the RDX nitroso-intermediates.

This study has contributed significantly to an explanation of how RDX, a nitramine explosive, is biodegraded under anaerobic conditions. Specifically, RDX is biodegraded by serving as a terminal electron acceptor, a fundamentally different mechanism than that used by bacteria for degrading other organic compounds. These findings have widespread application in wastewater treatment and cleanup technologies. Furthermore, this increased understanding of the mechanism used by bacteria to biodegrade RDX will be useful in identifying and isolating the requisite enzymes.

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